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Nitric Oxide Function in an Echinoderm

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Abstract. In vertebrates, nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS) and regulates relaxation of smooth muscle by activating the cyclic-GMP (cGMP) generating enzyme soluble guanylyl cyclase (SGC). Here we show that the NO-cGMP pathway mediates relaxation of the cardiac stomach in the starfish *Asterias rubens*. The NO-donors hydroxylamine, *S*-nitrosoglutathione (SNOG) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) and the NOS substrate L-arginine cause relaxation of the cardiac stomach. The relaxing effect of SNAP is blocked by the SGC inhibitor 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and the relaxing effect of L-arginine is inhibited by ODQ and the NOS inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA). ODQ and methylene blue also cause contraction, which may be due to inhibition of the relaxing action of NO produced by cells in the cardiac stomach. These results suggest that NO is synthesized in the cardiac stomach and regulates relaxation by activating SGC. NO-cGMP-mediated relaxation of the cardiac stomach may be important during feeding in starfish where the relaxed stomach is everted through an oral opening and over the digestible parts of prey. The discovery of NO-cGMP-mediated relaxation in an echinoderm demonstrates that regulation of smooth muscle tone by this signaling pathway also occurs in animals other than vertebrates.

Introduction

Nitric oxide (NO) is one of a class of gaseous chemicals that have been identified as signaling molecules in the nervous system (Dawson and Snyder, 1994). NO is synthesized in neurons by a Ca^{2+}/calmodulin-activated, NADPH-dependent enzyme known as nitric oxide synthase (NOS). NO diffuses from sites of synthesis into adjacent cells and exerts effects by activating the enzyme soluble guanylyl cyclase (SGC) to generate the second messenger cGMP (Garthwaite, 1991). This NO-cGMP signaling pathway appears to be evolutionarily ancient because it is present in a wide range of animal groups including cnidarians (Colasanti *et al.*, 1995; Salleo *et al.*, 1996), nematodes (Bascal *et al.*, 1995), annelids (Leake and Moroz, 1996), molluscs (Jacklet and Gruhn, 1994), arthropods (Müller, 1997), and vertebrates (Nilsson and Söderström, 1997). Moreover, diverse functions for the NO-cGMP pathway have been identified, including roles in learning and memory (Schuman and Madison, 1994; Robertson *et al.*, 1996), feeding (Colasanti *et al.*, 1995; Elphick *et al.*, 1995b; Salleo *et al.*, 1996), olfaction (Gelperin, 1994; Müller and Hildebrandt, 1995), and regulation of mammalian smooth muscle tone (Palmer *et al.*, 1987; Bult *et al.*, 1990).

One major invertebrate phylum in which the NO-cGMP signaling system has remained largely unexamined is the echinoderms. In fact, the only evidence of the existence of the NO-cGMP pathway in this phylum is an immunocytochemical study using an antiserum to rat brain NOS in which NOS-like immunoreactive neurons were detected in the cardiac stomach of the starfish *Marthasterias glacialis* (Martinez *et al.*, 1994). Interestingly, one of the functions of NO in vertebrates is to cause relaxation of smooth muscle in the gut (Olsson and Holmgren, 1997). The detection of NOS-like immunoreactivity in neurons of the starfish cardiac stomach suggests that NO may regulate muscle tone in this organ. The relaxing effect of NO on smooth muscle has been observed in a variety of vertebrate preparations, but it is not known whether NO has a similar role in invertebrates. Echinoderms occupy

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Abbreviations: NOS, nitric oxide synthase; SGC, soluble guanylyl cyclase; SNOG, *S*-nitrosoglutathione; SNAP, *S*-nitroso-*N*-acetylpenicillamine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; L-NMMA, N^{ω}-monomethyl-L-arginine.

an interesting position in animal evolution. Although they are invertebrates, echinoderms have a deuterostomian mode of development like the vertebrates. For this reason the echinoderms and a few other invertebrate deuterostomian phyla are recognized as being more closely related to vertebrates than the protostomian invertebrate phyla such as arthropods, molluscs, and annelids. Therefore, comparative analysis of NO function in echinoderms is of particular interest.

In the present study we have investigated the role of the NO-cGMP pathway in the cardiac stomach of the starfish *Asterias rubens*. To do this a variety of drugs that interact with components of the NO-cGMP signaling system were tested for their effects on the contractility of an *in vitro* preparation of the cardiac stomach. The results indicate that NO is involved in regulating the starfish cardiac stomach by causing relaxation of muscle tone. This effect of NO may be important during feeding in starfish where the relaxed cardiac stomach is everted through an oral opening and over the digestible parts of prey animals such as mussels and oysters.

Materials and Methods

Specimens of *Asterias rubens* were purchased from the University Marine Biological Station at Millport, Scotland, and kept in a seawater aquarium at Queen Mary and Westfield College. Cardiac stomach preparations were dissected and linked to a transducer as described by Elphick *et al.* (1995a). The preparation was suspended in a 20-ml organ bath containing aerated seawater maintained at 11°C and was then left to equilibrate for about 15 min. To investigate the effects of drugs that interact with the NO-cGMP pathway, the seawater (which contains about 10 m*M* K^+) was replaced with seawater containing 30 m*M* added KCl. These depolarizing conditions induce sustained but sub-maximal contracture of the cardiac stomach, making it possible to observe the effects of drugs that cause relaxation.

Cardiac stomach movement was recorded using a Harvard isotonic transducer (0.5 g load) linked to a Harvard twin-channel Universal oscillograph. A range of gain settings on the oscillograph were used depending on the responsiveness of the preparation. Each of the recordings illustrated in the figures are accompanied in the figure legend with the gain setting to enable comparison of responses.

Drugs tested included the NO-donors hydroxylamine, *S*-nitrosoglutathione (SNOG) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). *N*-acetylpenicillamine (NAP) was used as a negative control for SNAP to establish whether effects observed could be attributed to SNAP's ability to release NO. The substrate for NOS, L-arginine, was tested using D-arginine as a negative control. Test compounds that inhibit NO-cGMP signaling included methylene blue, an inhibitor of both NOS and SGC (Miki *et al.*, 1987; Mayer *et al.*, 1993); the NOS inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA) (Moncada *et al.*, 1991); and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a compound recently identified as a selective inhibitor of SGC (Garthwaite *et al.*, 1995).

All of the drugs were tested on at least three different preparations and representative responses are illustrated in the figures. Where enzyme inhibitors were used, the percent inhibition was calculated from three or more experiments as the mean ± standard error of the mean.

Drugs were obtained from Sigma (Poole, Dorset, UK) except SNAP, SNOG, and L-NMMA, which were gifts from the Wellcome Research Laboratories, and ODQ, which was purchased from Tocris Cookson (Bristol, UK). Drugs were prepared as aqueous solutions except ODQ, which was dissolved in DMSO and diluted in water with the final concentration of DMSO in the organ bath not exceeding 0.12%. Drugs were added to the organ bath in volumes of 10–100 μl to achieve the bath concentrations shown in the figures.

Results

The NO-donors hydroxylamine, SNOG, and SNAP caused relaxation of the cardiac stomach (Fig. 1). The relaxing effect of SNOG (10 μM) was rapidly reversed on washing (Fig. 1b), whereas hydroxylamine (1 m*M*) and SNAP (10 μM) had longer lasting effects as several washes were required before basal tone was restored (Fig. 1a, c). Differences in the reversibility of the relaxing effects of the NO donors may reflect their relative permeability in cardiac stomach tissue. The magnitude of the relaxing effect of the NO-donors was dose-dependent as illustrated for SNAP in Figure 1c. If three chemically unrelated NO-donors cause relaxation of the cardiac stomach, then it is likely that their effects are attributable to their ability to release NO rather than to some other chemical property of these compounds. Nevertheless, we addressed this question by testing NAP which, except for the absence of an NO moiety, is otherwise chemically identical to SNAP. NAP did not cause relaxation of the cardiac stomach when tested at a concentration (10 μM) at which SNAP causes marked relaxation (Fig. 1d).

Methylene blue caused dose-dependent contraction of the cardiac stomach (Fig. 2a). However, this compound inhibits both NOS and SGC, which makes interpretation of results difficult. ODQ, a selective inhibitor of SGC, has low solubility in water, and DMSO was used as a solvent to prepare stock solutions. In some preparations, however, the DMSO-containing vehicle had a contracting effect on the cardiac stomach. Therefore, we have illustrated here experiments in which the DMSO-containing

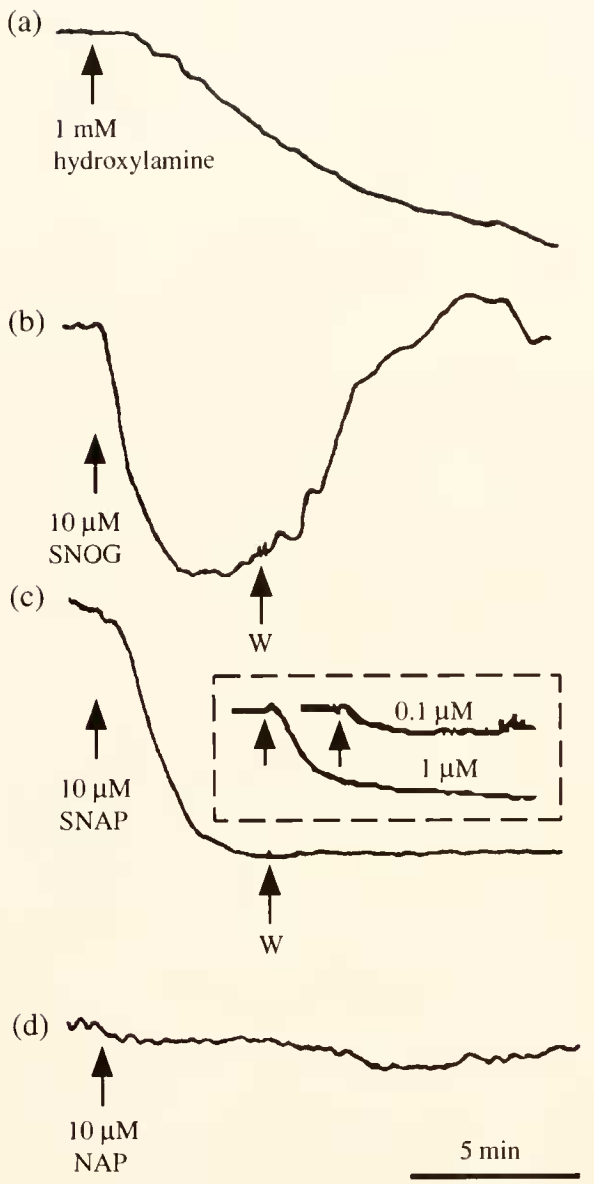


Figure 1. NO-donors cause relaxation of the cardiac stomach. (a) Hydroxylamine; gain 1. (b) SNOG; gain 5. (c) SNAP; gain 7. (d) NAP, which is chemically identical to SNAP apart from the absence of a NO moiety, does not cause relaxation; gain 5. [W = wash] In (c) and (d) there is a slight decrease in baseline tension during the course of the experiments, but the responses to SNAP and NAP, respectively, are clearly very different.

vehicle had no effect on muscle tone. Doses of 1 μM and 3 μM ODQ had no effect on cardiac stomach tone, but 10 μM ODQ consistently caused contraction (Fig. 2b). However, the delay in the onset of contraction and the rate of contraction with 10 μM ODQ were quite variable in different preparations.

The contractions caused by ODQ on the cardiac stomach suggest that ODQ is exerting this effect through inhibition of SGC activation by endogenous NO. To investigate the mode of action of ODQ on the cardiac stomach, we tested ODQ in combination with exogenous NO released by the NO-donor SNAP. First SNAP was tested alone at a concentration (10 μM) at which it consistently causes relaxation of the cardiac stomach (Fig. 3a). Then, after washing, the cardiac stomach was incubated with 10 μM ODQ for 15 min before applying a second dose of 10 μM SNAP (Fig. 3b). ODQ caused almost complete inhibition (94.8% in Fig. 3b) of the relaxing effect of SNAP. After washing out of the ODQ, SNAP was tested a third time and in the absence of ODQ the normal relaxing effect of 10 μM SNAP was restored (Fig. 3c). The mean percentage inhibition by 10 μM ODQ on the relaxing effect of 10 μM SNAP in five experiments on different preparations was 88.7 ± 2.3% (Fig. 3d). A mean inhibition of 56.6% was observed in two other experiments using 3 μM ODQ (data not shown).

The experiments described so far indicate that NO causes relaxation of the cardiac stomach by activating SGC. In the presence of the SGC inhibitor ODQ, the relaxing effect of endogenous NO is blocked, leading to contraction. However, this provides only indirect evi-

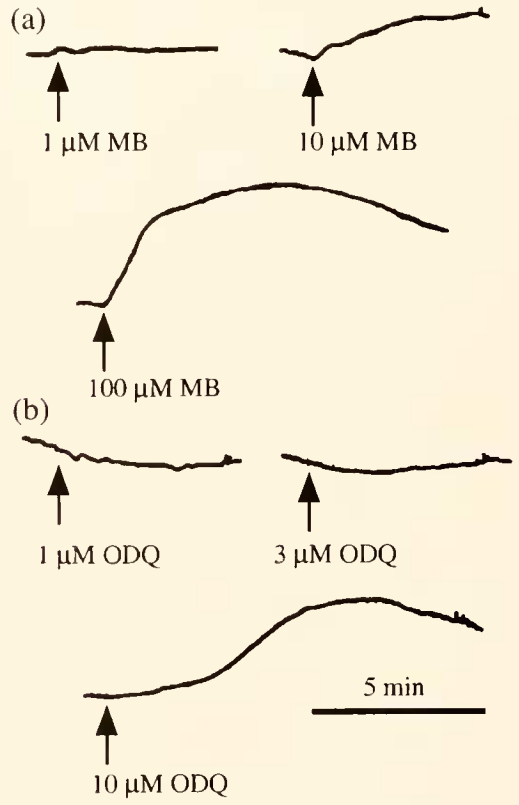
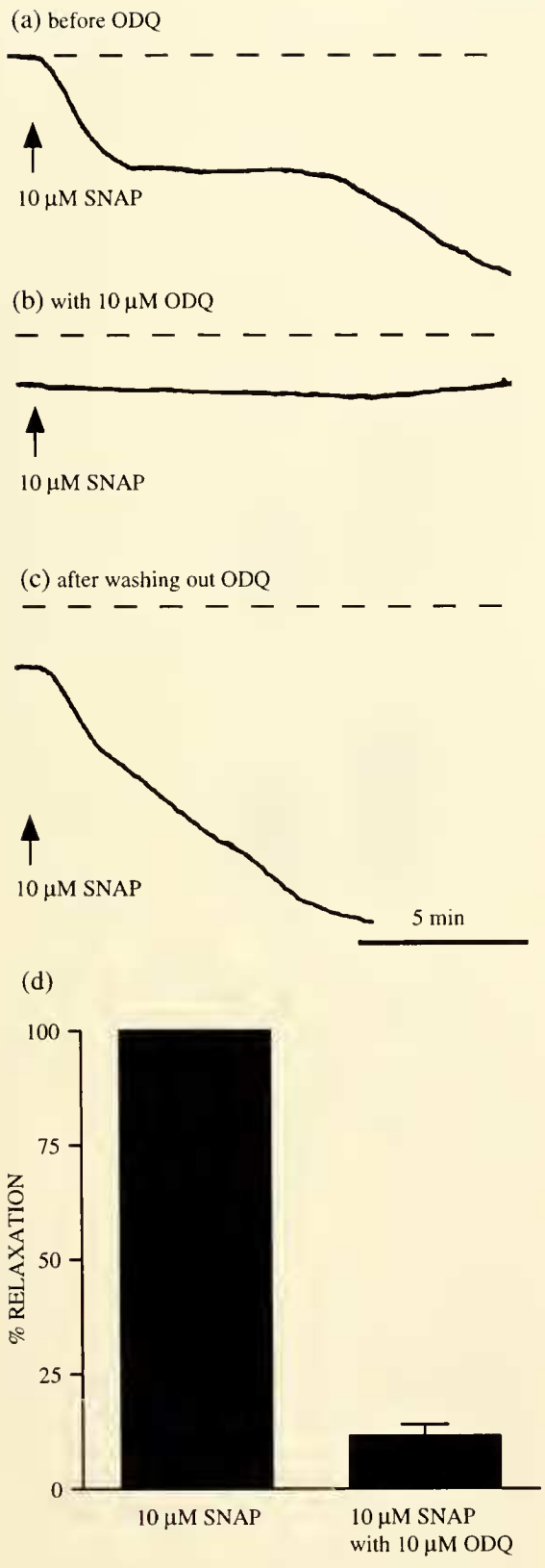


Figure 2. The NO-cGMP pathway inhibitors methylene blue and ODQ cause contraction of the cardiac stomach. (a) Methylene blue (MB); gain 5. (b) ODQ; gain 4.



dence that NO is synthesized and released by cells in the cardiac stomach. We tested for NO production by NOS in the cardiac stomach by examining the effects of its substrate L-arginine. We also tested the NOS inhibitor L-NMMA.

L-arginine caused relaxation of the cardiac stomach when tested at 0.1 m*M* and 1 m*M*. In the experiments illustrated in Figure 4a and b, first the D-isomer of arginine was tested at 0.1 m*M*; this had no effect on muscle tone (Fig. 4a), whereas the L-isomer tested at the same concentration caused a marked relaxation of the cardiac stomach (Fig. 4b). At 1 m*M*, however, D-arginine sometimes caused slight relaxation of the cardiac stomach. This may be due to the presence of small amounts of L-arginine in our D-arginine stock. Alternatively, D-arginine may produce effects at 1 m*M* due to the action of an isomerase that converts D-arginine to L-arginine.

In each preparation, consistent dose-dependent relaxation was observed with L-arginine. However, preparations varied in the shape and magnitude of their responses to L-arginine. For example, some preparations relaxed rapidly and then partially restored basal tone prior to washing (Fig. 4b) and others responded with a slow but sustained relaxation that was only reversed on washing (Fig. 4c). Differences in responses among preparations probably reflect natural variability in the motility and metabolic state of the cardiac stomach in starfish.

L-NMMA did not have a consistent effect on cardiac stomach tone when tested alone, although we observed a gradual and slight increase in tone in some preparations. However, in preparations pre-incubated with L-NMMA for 30 min, the relaxing effect of L-arginine (Fig. 4c) was partially inhibited (56%; Fig. 4d). The mean percentage inhibition of 1 m*M* L-arginine-induced relaxation by 0.1 m*M* L-NMMA from three experiments performed on different preparations was 60.0 ± 9.9% (Fig. 4e).

We further investigated the mode of action of L-arginine on the cardiac stomach by testing the effect of ODQ on L-arginine responses. Ten μM ODQ caused partial inhibition (41.4%) of the relaxing effect of L-arginine (Fig. 4f) as illustrated in Figure 4g. In three experiments

Figure 3. ODQ inhibits SNAP-induced relaxation of the cardiac stomach. (a) SNAP-induced relaxation before application of ODQ. (b) Incubation in 10 μM ODQ for 15 min prior to application of SNAP inhibits (94.8%) the relaxing effect of SNAP. (c) After washing (60 min), the relaxing effect of SNAP is restored. The dashed line in each section of the figure indicates basal tone in the preparation prior to application of the first sample of SNAP in (a). The biphasic nature of the response to SNAP in (a) but not in (c) was not seen in all preparations tested, but we have shown it here because it reflects natural variability that is typical for this preparation. The gain is 2 in (a), (b), and (c). (d) Graph showing the mean and standard error of the inhibitory effect of ODQ on SNAP-induced relaxation from five experiments on different preparations.

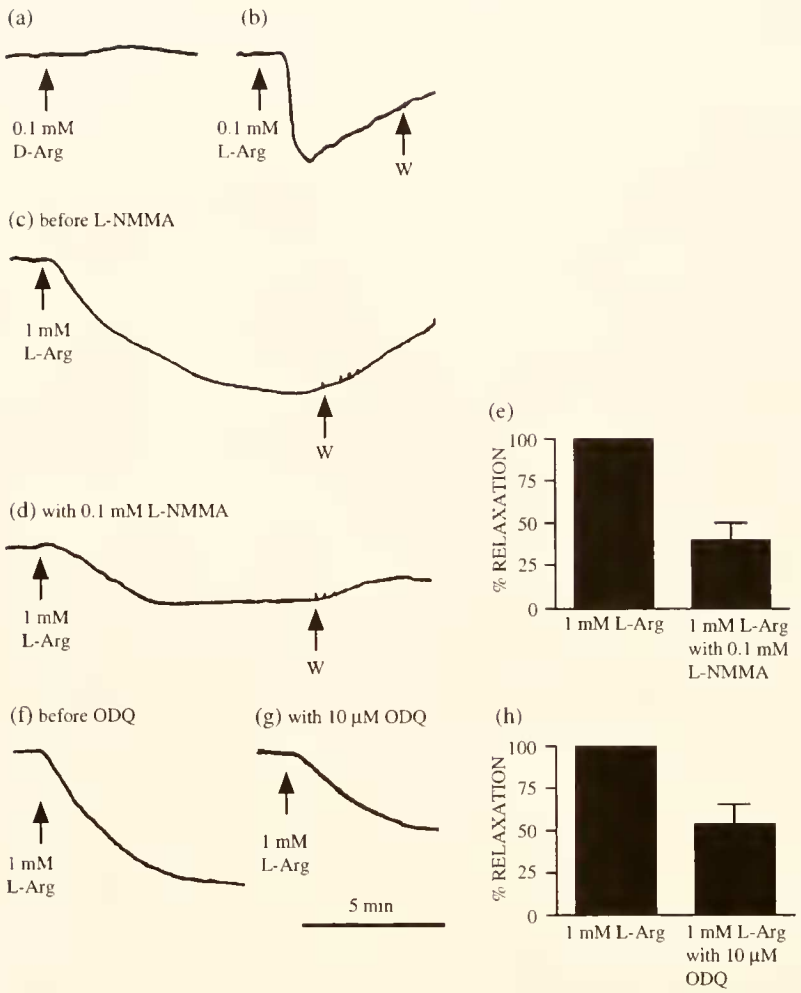


Figure 4. L-NMMA and ODQ inhibit L-arginine-induced relaxation of the cardiac stomach. (a) D-arginine has no effect on a cardiac stomach preparation in which (b) L-arginine causes relaxation; gain 5. (c) L-arginine-induced relaxation is (d) inhibited (56%) when the preparation is pre-incubated with 0.1 m*M* L-NMMA for 30 min; gain 3. (e) Graph showing the mean and standard error of the inhibitory effect of 0.1 m*M* L-NMMA on 1 m*M* L-arginine-induced relaxation from three experiments on different preparations. (f) L-arginine-induced relaxation is (g) inhibited (41.4%) when the preparation is pre-incubated with 10 μM ODQ for 15 min; gain 3. (h) Graph showing the mean and standard error of the inhibitory effect of 10 μM ODQ on 1 m*M* L-arginine-induced relaxation from three experiments on different preparations.

performed on different preparations, the mean percentage inhibition of 1 m*M* L-arginine-induced relaxation by 10 μM ODQ was 46.5 ± 11.6% (Fig. 4h).

Discussion

The results of the experiments described here demonstrate that NO causes relaxation of the starfish cardiac stomach and indicate the presence of a NO-cGMP signaling pathway in the cardiac stomach. Three chemically distinct NO-donors, hydroxylamine, SNOG, and SNAP, caused relaxation of the cardiac stomach while NAP, which is chemically identical to SNAP apart from the absence of a NO moiety, had no effect on the cardiac stomach. This provides clear evidence that NO causes relaxation of the starfish stomach. However, it is more difficult to demonstrate that NO is naturally released by cells in the cardiac stomach to regulate muscle tone. Nevertheless, by testing a range of NO-cGMP pathway inhibitors and the substrate for NOS, L-arginine, we have obtained a series of consistent responses, all of which support the notion that NO is a natural regulator of muscle contractility in the starfish cardiac stomach.

Methylene blue causes contraction of the cardiac stomach in a dose-dependent manner, but we cannot be certain of the mode of action of this compound because it inhibits

both of the key enzymic components of the NO-cGMP pathway, NOS and SGC (Miki *et al.*, 1987; Mayer *et al.*, 1993). Therefore, inhibition of basal NO production by NOS or inhibition of the activation of SGC by endogenous NO could account for the contracting effect of methylene blue on the cardiac stomach. The effects of ODQ on the cardiac stomach, however, can be attributed to its property as a selective inhibitor of SGC (Garthwaite *et al.*, 1995). ODQ causes contraction of the cardiac stomach, suggesting that it is blocking activation of SGC by endogenous NO. Evidence that ODQ exerts its effects by inhibiting activation of SGC by endogenous NO is provided by experiments in which ODQ blocked the relaxing effect of exogenous NO in the form of the NO-donor SNAP. ODQ also partially inhibits the relaxing effect of the NOS substrate L-arginine, indicating that endogenous production of NO regulates cardiac stomach tone by activating SGC. Likewise, the NOS inhibitor L-NMMA partially inhibits the relaxing effect of L-arginine, indicating the presence of NOS in the cardiac stomach.

Therefore, collectively the experiments described here indicate that NO is synthesized by NOS in the cardiac stomach and regulates this organ by activating SGC to generate cGMP, which leads to relaxation. The cellular location of the enzymic components of this NO-cGMP pathway in the cardiac stomach is not known. However, the presence of NOS-like immunoreactivity in basiepithelial neurons of cardiac stomach (Martinez *et al.*, 1994) suggests that NO is released by neurons. It seems most likely that NO is released in the basiepithelial nerve plexus, which is separated in the cardiac stomach from a smooth muscle layer by a thin layer of connective tissue (see Moore and Thorndyke, 1993, for diagrams). The most likely location for SGC is in the smooth muscle cells of the cardiac stomach, as in the mammalian gut (Boeckxstaens and Pelckmans, 1997). Here activation of SGC by NO would cause increased cGMP levels, which in turn could act as an intracellular signal mediating relaxation.

The proposed anatomy of NO-cGMP signaling in the cardiac stomach may explain some of the results obtained with NO-cGMP pathway inhibitors. The SGC inhibitor ODQ caused contraction of the cardiac stomach. We attribute this to inhibition of SGC activation by endogenously released NO. If this is correct, then one could predict that the NOS inhibitor L-NMMA would also cause contraction by inhibiting endogenous NO production. However, with L-NMMA we did not observe contractions like those seen with ODQ, although in some preparations a gradual increase in tone was observed. How can this be explained? It may simply reflect the relative permeability of the two drugs in cardiac stomach tissue and the depth of tissue they must penetrate to reach their molecular targets. The molecular target of ODQ (SGC) is likely to be located in the superficial muscle layer, whereas the molecular target of L-NMMA (NOS) is apparently located in neurons (Martinez *et al.*, 1994) that are separated from the bathing medium by several layers of cells and connective tissue.

It should be recognized, however, that although the function of the NO-cGMP pathway in the starfish cardiac stomach may be similar to its role in the mammalian gut, the anatomy of the innervation is quite different. In mammals, NO is released by inhibitory non-adrenergic non-cholinergic (NANC) nerves of the peripheral autonomic nervous system (Boeckxstaens and Pelckmans, 1997). Homologs of NANC nerves are not present in echinoderms, and innervation of the starfish cardiac stomach consists solely of an intrinsic basiepithelial nerve plexus. Moreover, NO is only one of a number of molecules that are considered to function as inhibitory NANC neurotransmitters in mammals. Other substances that may act in series or in parallel with NO include the neuropeptide vasoactive intestinal peptide and ATP (Boeckxstaens and Pelckmans, 1997).

The discovery of a NO-cGMP signaling system in the starfish cardiac stomach is interesting for a number of reasons. This is the first study, to the best of our knowledge, that has investigated NO function in an echinoderm. Moreover, NO-cGMP-mediated relaxation of smooth muscle has thus far been described only in vertebrates. The presence of this pathway in a stomach preparation from an echinoderm indicates that NO-cGMP-mediated smooth muscle relaxation may be widespread in the animal kingdom.

The presence of the NO-cGMP pathway in the starfish cardiac stomach is of particular interest because of the role this organ plays in the unusual feeding behavior of these animals. Starfish like *Asterias rubens* feed by everting their cardiac stomach through an oral opening and over digestible parts of prey such as mussels and oysters. Very little is known about how eversion of the cardiac stomach is controlled, but it is clear that the stomach must be relaxed for eversion to be accomplished (Anderson, 1954). Recently, a neuropeptide (S2) belonging to a family of echinoderm peptides known as SALMFamides was identified as a potent relaxant of the starfish cardiac stomach (Elphick *et al.*, 1991, 1995a). In the present study we show that the NO-cGMP pathway also mediates relaxation of the cardiac stomach. This indicates that cardiac stomach relaxation and eversion is controlled by several neuronal signaling systems that may act in parallel or in series. The complexity of the pharmacology of the cardiac stomach is therefore comparable with the aforementioned NANC innervation of the mammalian gut. In the future we plan to investigate possible interactions between the SALMFamide neuropeptide system and the

NO-cGMP pathway in regulating relaxation of the starfish cardiac stomach.

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